IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Choong-Chin Liew	Examiner:	Juliet Switzer
Serial No.:	10/812,737		
Filed:	March 30, 2004	Group Art Unit:	1634
Titled:	Method for the Detection of Bladder Cancer Related Gene Transcripts in Blood		
		Conf. No.:	4510

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF Hongwei Zhang UNDER 37 C.F.R. §1.132

Sir:

I, **Hongwei Zhang**, Ph.D., hereby declare that:

1. I received a Ph.D. degree from the Institute of Medical Science at the University of Toronto in 2002, and a Master of Science degree from the Department of Immunology at the University of Toronto in 1995. In addition I received my Medical Degree from the University of Medical Sciences in Changchun China in 1989 and practiced as a staff physician for 4 years in Beijing prior to commencing my post graduate studies. I currently hold the position of Director of Biomarker Development at GeneNews Corporation (formerly ChondroGene Ltd., the Assignee of the application).

I am a trained molecular biologist experienced in developing methods to identify biomarkers which are indicative of a disease or condition, and in developing methods of using these biomarkers and products thereof as applied in the area of bladder cancer, amongst other conditions.

List of Publications:

K.W. Marshall, M.D., PhD., F.R.C.S., **H. Zhang, M.D., PhD.**, T.D. Yager Ph.D., N. Nossova M.D., Ph.D., A. Dempsey PhD., R. Zheng M.D., M. Han M.D. Ph.D., H.Tang M.Sc., S. Chao M.A.Sc, and C.C. Liew PhD. "Blood-based biomarkers for detecting mild osteoarthritis in the human knee" *OsteoArthritis and Cartilage (2005)* 861-871.

Zhang H, Marshall KW, Tang H, Hwang DM, Lee M, Liew CC. Profiling genes expressed in human fetal cartilage using 13,155 expressed sequence tags. Osteoarthritis Cartilage 2003;11:309-19.

Hongwei Zhang, C.C.Liew, K.Wayne Marshall. Microarray Analysis Reveals the Involvement of Beta-2 Microglobulin (B2M) in Human Osteoarthritis. Osteoarthritis and Cartilage 2002;10:950-60.

Doherty PJ, **Zhang H**, Manolopoulos V, Trogadis J, Tremblay L, Marshall KW. Adhesion of transplanted chondrocytes onto cartilage in vitro and in vivo. J Rheumatol 2000;27:1725-312.

Zhao YX, Lajoie G, **Zhang H,** Chiu B, Payne U, Inman RD. Tumor necrosis factor receptor p55-deficient mice respond to acute Yersinia enterocolitica infection with less apoptosis and more effective host resistance. Infect Immun 2000;68:1243-513.

Vaselios Manolopoulos, K. Wayne Marshall, **Hongwei Zhang**, Judy Trogadis, Louise Trembly and Paul J. Doherty. Factors affecting the efficacy of bovine chondrocyte transplantation in vitro. Osteoarthritis and Cartilage 1999;7:453-460.

Yi-Xue Zhao, **Hongwei Zhang**, Basil Chiu, Usulra Payne, Robert D. Inman. Tumor necrosis factor receptor P55 controls the severity of arthritis in experimental *Yersinia Enterocolitica* infection. Arthritis & Rheumatism 1999;42:1662-1672.

Paul J. Doherty, **Hongwei Zhang**, Louise Trembley, Vaselios Manolopoulos and K. Wayne Marshall. Resurfacing of articular cartilage explants with genetically-modified human chondrocytes *in vitro*. Osteoarthritis and Cartilage 1998;6:153-160.

Hongwei Zhang, Donna Phang, Ronald M. Laxer, Earl D. Silverman, Sueihua Pan, and Paul J. Doherty. Evolution of the T cell receptor beta repertoire from synovial fluid T cells of patient with juvenile onset rheumatoid arthritis. J. Rheumatol. 1997;24:1396-402.

Petro Lastres, Anihoa Letamendia, **Hongwei Zhang**, Carlos Rius, Nuria Almendro, UIIa RAab, Louis A. Lopez, Carmen Langa, Angels Fabra, Michelle Letarte and Carmelo Bernabeu. Endoglin modulates cellular responses to TGF-beta 1. J. Cell Biol. 1996;133:1109-1121.

Hongwei Zhang, Andrew R.E. Shaw, Allan Mak, and Michelle Letarte. Endoglin is a component of the Transforming Growth Factor (TGF)-beta receptor complex of human pre-B leukemic cells. J. Immunol. 1996, 156:565-573.

2. I have read the non-final Office Action mailed May 11, 2007 in the above-referenced patent application.

In providing grounds for rejection of claims under 35 U.S.C. § 112(1), the Examiner asserts at page 8 of the Office Action: "*It is not known under what circumstances the result observed in the instantly examined control and test populations would be repeatable, as the results have not been validated.*"

3. As a scientist skilled in the area of molecular biomarker identification, I submit that post-filing validation experiments performed by the Assignee of the present application using both quantitative RT-PCR (QRT-PCR), an alternate technology relative to microarray analysis employed in the experiments disclosed at Example 19 of the specification, as well as an independent cohort of control and disease subjects relative to those employed in the experiments disclosed at Example 19 of the specification, have shown that RNA encoded by the gene ADAM9 is present at statistically different levels in blood of subjects having bladder cancer relative to healthy control subjects.

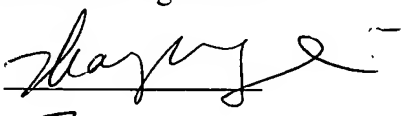
<u>Levels of ADAM9 expression in blood are statistically different in bladder cancer patients versus healthy control subjects – validation of ADAM9 as biomarker of bladder cancer in blood via an alternate technology (quantitative RT-PCR) using an independent cohort</u>

Attached as Exhibit "A" to this Declaration are results and materials and methods of post-filing experiments performed by the Assignee of the present application in which levels of ADAM9-encoded RNA in blood were found to be statistically different in subjects having bladder cancer relative to healthy control subjects, as determined via QRT-PCR, an alternate technology relative to the microarray analysis employed in the experiments disclosed at Example 19 of the specification, and with an independent cohort of control and disease subjects relative to that described at Example 19 of the specification. As shown in Tables 1 and 2 of Exhibit "A", the average level of ADAM9-encoded RNA in blood samples from 16 bladder cancer patients, as determined via $\Delta\Delta$Ct values obtained from QRT-PCR analysis, was found to be significantly different relative to that of 21 healthy

control subjects tested, with the difference in expression levels being statistically significant (p=0.032).

In view of the above, I submit that the specification enables one of skill in the art to practice the claimed methods.

5. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that wilful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

Hongwei Zhang, Ph.D. 

Date Nov. 23, 2007

EXHIBIT "A"

TABLE 1. Quantitative RT-PCR analysis of ADAM9-encoded RNA levels in blood of bladder cancer patients versus healthy control subjects.

Experimental group	Sample ID	Relative fold-change
Healthy control	N25Cp	0.857
	N56p	0.958
	N68p	1.171
	N69p	1.600
	N70p	1.359
	N71p	0.754
	N72p	1.472
	N73p	0.602
	N74p	0.843
	N75p	0.888
	N76p	1.081
	N77Ap	0.912
	N78Ap	1.123
	N79Ap	1.002
	N80Ap	0.866
	N81Ap	1.447
	CL-B	1.251
	CL-C	0.822
	CL-D	1.070
	IO055p	0.786
	CL08p	0.794
Bladder cancer	CL09	0.015
	CL09p	0.533
	CL56	1.572
	IO010p	0.588
	IO013p	1.063
	IO022p	1.030
	IO044p	0.575
	IO045Bp	0.817
	IO046p	0.897
	IO047p	0.828
	IO048p	0.781
	IO049p	0.535
	IO050p	0.489
	IO051p	0.840
	IO052Bp	1.341
	IO052p	0.888

TABLE 2. Analysis of QRT-PCR data of Table 1, above, for bladder cancer detection.

Relative average level of ADAM9 expression (raw Ct)	**healthy control subjects**	1.0
	bladder cancer patients	0.80
Average fold-change ADAM9 expression (bladder cancer patients/control subjects)		0.80
***p*-value**		0.032

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***p*-value**		0.032

Materials and Methods:

Blood RNA Isolation: Samples were obtained from 16 patients diagnosed with bladder cancer, and from 21 healthy control subjects. All participants provided written informed consent. Approximately 10ml of blood was collected from each participant, using a Vacutainer™ tube (Becton Dickinson, Franklin Lakes, NJ). Red blood cells were ruptured with hypotonic lysis buffer (1.6 mM EDTA, 10 mM KHCO3, 153 mM NH4Cl, pH 7.4), followed by collection of white blood cells by centrifugation. White blood cell total RNA was extracted with Trizol® Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. The quality of RNA samples was assessed on an Agilent Bioanalyzer 2100 using RNA 6000 Nano Chips (Agilent Technologies, Palo Alto, CA), and the quantities of RNA were measured by UV spectrophotometry (Beckman-Coulter DU640).

Real-time QRT-PCR: Real-time QRT-PCR was used to measure levels of ADAM9-encoded RNA in blood samples from bladder cancer patients and healthy control subjects. First strand cDNA was synthesized from 1μg total RNA using the ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in a volume of 100μl, consisting of 10μl 10x RT buffer, 4μl 100mM dNTP mix, 10μl 10x RT random primers and 5μl Multi-scribe reverse transcriptase (50U/μl). Real-time PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). PCR was performed in a reaction volume of 25μl consisting of 12.5μl 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5μl of 5pmol primer mix (sense primer, TGCCACTGGGAATGCTTTGTGT; anti-sense primer, CCAACATTTGGTGCCTCGACTA; both primers are optimized for an annealing temperature of 56°C), and 2.5ng first strand cDNA. The PCR cycling protocol used is as follows: (1) 50°C, 2 min; (2) 95°C, 10 min; (3) 40 cycles of 95°C, 15 sec; 60°C, 1 min; and (4) determining the dissociation curve from 60°C to 95°C. The gene GAPDH was used as the housekeeping gene for normalization.

Fold-change/statistical analysis: A Welch's t-test was applied to the fold-change values to test for the statistical significance of the difference in RNA levels between the disease and healthy control groups. Fold change was calculated using the following

formula: 2-ΔΔCt, where ΔΔCt was calculated by subtracting the mean ΔCt value of the control samples from the ΔCt of each sample for each gene. Statistical analysis was performed using SigmaStat v3.0 (SPSS Scientific, Chicago, IL).